VOLATILE ORGANIC BIOMARKERS IN EXHALED BREATH AS A RAPID, PRODROMAL, DIAGNOSIS OF BIOAGENT INFECTION

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ABSTRACT

We have studied the feasibility of using biomarkers in exhaled breath as a prodromal diagnostic for bioagent infection. A compact sampling system was developed and used to collect more than 75 breath samples from 9 piglets. Three of the sampled piglets were uninfected control animals, three were infected with staphylococcal enterotoxin B (SEB), and three with *Actinobacillus pleuropneumoniae* (*A.p.*). We found that: volatile markers appeared several hours before outwardly noticeable physical symptoms of illness; volatile profiles for infected piglets were distinct from the uninfected animals and room background air samples; and the volatile profiles for SEB and *A.p.* were different.

INTRODUCTION

Trace organic compounds in expired human breath reflect both physiological state and exposure to the surrounding environment and therefore provide a useful index of disease.[1-6] As discussed below, molecular markers that indicate specific diseases are known for several diseases, both infectious and noninfectious. As far as we know, the question has not been investigated for BW-agent-induced diseases, however, by logical extension, signature compounds should be present. The breath analysis of VOCs opens a noninvasive window to normal metabolic pathways, and also illustrates how these pathways are altered by disease.

Human breath can contain many hundreds of VOCs in low concentrations although fewer than fifty of these are found at any one time in the majority of normal humans.[5] Many of these are emitted under normal physiological circumstances, and others only when disease is present. The latter can be new compounds produced by the invader and/or the host, or elevated levels of those normally present. For example, methylated alkanes are normally present in breath but rise sharply due to increased lipid peroxidation associated with elevated levels of reactive oxygen species (ROS) arising from an active disease state.[3-6] Toxic VOCs can also be found in exhaled breath after inhalation of polluted air. Many studies have been performed on this last topic, in large part because the compounds are present at relatively high concentrations. In contrast, few studies have been conducted on infectious disease-produced organic compounds, which may be present at very low concentrations, particularly very soon after infection.

Breath analysis has a long history of application to detection of disease.[7] Before the development of modern analytical instrumentation, doctors used qualitative descriptions of breath odor to diagnose disease. Examples of fruity odors, indicating diabetes, and fishy smells, indicating uremia, are well

1. REPORT DATE **16 NOV 2004**	2. REPORT TYPE **N/A**	3. DATES COVERED **-**	
4. TITLE AND SUBTITLE **Volatile Organic Biomarkers In Exhaled Breath As A Rapid, Prodromal, Diagnosis Of Bioagent Infection**		5a. CONTRACT NUMBER	
		5b. GRANT NUMBER	
		5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)		5d. PROJECT NUMBER	
		5e. TASK NUMBER	
		5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025**		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)	
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release, distribution unlimited**			
13. SUPPLEMENTARY NOTES **See also ADM001849, 2004 Scientific Conference on Chemical and Biological Defense Research. Held in Hunt Valley, Maryland on 15-17 November 2004., The original document contains color images.**			
14. ABSTRACT			
15. SUBJECT TERMS			

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT **UU**	18. NUMBER OF PAGES **7**	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**			

known, and terms for breath odors have found their way into common medical vocabulary, such as uremic breath, diabetic breath, and fetor hepaticus.[8]

With modern instrumentation, relationships between disease and specific molecules in breath have been obtained. Examples of specific molecular markers associated with different diseases are listed in Table 1. Note that although some of the diseases are associated with the mouth or lungs, other diseases, such as diabetes, schizophrenia, or breast cancer, do not involve the respiratory system. Thus, it is not essential that the disease-specific markers occur in the respiratory system to provide a marker observable through breath analysis.

TABLE 1. Molecular Markers Associated With Specific Diseases

Disease/Biological Process	Marker(s)	Reference
Diabetes	Acetone	9,10
Uremia	Dimethylamine, trimethylamine	11
Cirrhosis	Dimethyl sulfide, mercaptans, fatty acids, ammonia	12,13
Halitosis	Methanethiol	14
Periodontal disease	Pyridine and picolines	15
Lung cancer	Alkanes, ketones, and benzene derivatives	7, 16, 17
Reperfusion after myocardial infarction	Pentane	18
Breast cancer	Pentanes	19
Schizophrenia	Pentane and carbon disulfide	20
Trimethylaminuria	Trimethylamine	21
Ovulation	1-dodecanol, methanethiol, sulfides	8

Over the past few years it has become evident that for an invading infectious agent to be successful in establishing an infection, the host has to be receptive.[22-24] In being so, the host cell produces under the "command" of the invading organism a whole array of new cellular components that will aid in the establishment of the infection. Also, in the presence of the host cell, many bacterial virulence genes are induced. Therefore, the detection of host-mediated volatile metabolites is an indirect, but very powerful, way to detect the presence of an infectious agent.

It is well known that for an infection to be established, a unique association is required between the invading pathogen and the host. Such an association, in general, is tissue-specific. For instance, pathogenic *Escherichia*, *Shigella*, and *Salmonella* species are most commonly isolated from the intestines of humans and animals. Wound infections due to *Pseudomonas aeruginosa* are prevalent in burn patients, and the causative agent of strep throat is *Streptococcus pyogenes*. It is also known that there is an interactive play between the incoming pathogen and the host cell, resulting in a two-way signaling process with changes in gene expression on both sides.

Once an infection is established by the infectious agent, growth will be initiated. A typical growth curve will be established which may take a few hours or a few days, depending on the pathogen. An initial lag phase (no growth but active metabolism occurs) precedes the exponential growth phase, which precedes the stationary growth phase (no more cell division due to depletion of growth factors, oxygen, etc.). VOCs may be produced during any of the three phases in the growth curve. It is assumed that the tissue specificity of many pathogens will result in a unique and reproducible profile of VOCs and that the effect of diet or inborn errors in the metabolism of the host will be minimal or nonexistent. In addition, clinical symptoms are usually independent of the diet, life style, or inborn errors in metabolism.

Staphylococcal enterotoxins (SE) were originally recognized for their ability to induce the emesis and diarrhea that is associated with staphylococcal food poisoning.[25-31] A number of variants of SEs have been identified, with SEB being of major interest to the military as a threat agent, since it was identified as having been made into a weapon. SEs have been characterized as members of a family of related toxins whose potent biologic activities can lead to lethal shock and include pyrogenicity, enhancement of

endotoxic shock, immunosuppressive properties and mitogenesis of lymphocytes.[26,28,32,33] SEs are among a class of immunostimulatory molecules designated "superantigens" based on their ability to stimulate T-cells as a result of their simultaneous binding of major histocompatibility complex (MHC) class II receptors on antigen presenting cells and T-cell receptors expressing appropriate variable elements.[34] These receptor interactions lead to activation of lymphocytes, resulting in production of cytokines, lymphokines, and other mediators contributing to toxic shock. Full understanding of the molecular, biochemical, and cellular basis for the pathologic events mediated by staphylococcal enterotoxins remains unresolved; however, it is evident that several cell types and organ systems are involved in manifestation of SE toxic activities.[25,26,28,35-38]

SEB is a Class B biothreat agent, and we have applied breath analysis as a presymptomatic diagnostic for its exposure in the animal model. There are many biothreat agents that, in the end, result in lethal shock (e.g., *B. anthracis*, *Y. pestis*, other toxins), and studies to identify early indicators that signal eventual onset of vascular collapse from biothreat exposure could be undertaken. However, this work focused solely on SEB- and *Actinobacillus pleuropneumoniae*-induced release of VOCs in a time-dependent manner after exposure. The system we employed sampled breath multiple times before exposure, and then from early prodromal stages through each stage of the increasingly severe illness.

EXPERIMENTAL METHOD

Piglets were chosen as the test subjects based on the similarities between their respiratory system and that of humans. For the biomarker identification task, we chose to use a conventional GC/MS analytical method. To facilitate breath collection from the piglets, we developed a compact sampling system based on commercially available SPME fibers. These devices consist of a retractable wire coated with a thin film of one of four different active sorbent media: 75 µm carboxen/polydimethylsiloxane (low molecular weight compounds, gases); 100 µm polydimethylsiloxane (volatiles); 65 µm polydimethylsiloxane/divinylbenzene (volatiles, amines, nitroaromatics); and 70 µm carbowax/divinylbenzene (alcohols, polar compounds). A fixture was developed to securely hold one of each type of sampler in the exhaled breath stream of a piglet whose mouth and snout were inserted though the opening of a veterinary anesthesia mask. A photograph of the sampling system is shown in Figure 1.
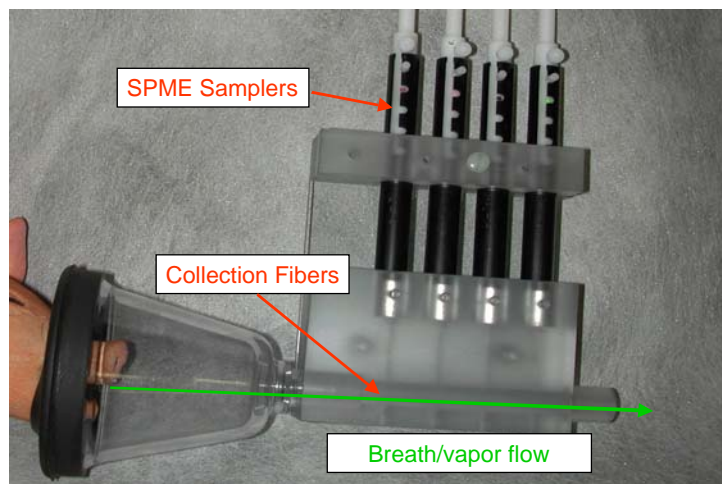

Figure 1. Collection of exhaled breath from a piglet test subject using SPME fibers.

Using the sampler described above, we collected more than 75 breath samples from 9 piglets. All sampling was performed at WRAIR. Three of the sampled piglets were uninfected control animals, three were infected with 150 µg/kg SEB, and three with 1×10^8 CFU *A.p.* The sampler was used to collect 30-minute breath samples before the pigs were infected and 30-minute breath samples at 60-minute intervals after infection. All samples were returned to SRI for analysis.

RESULTS AND DISCUSSION

SPME fibers were analyzed using an Agilent 5890 Series II GC equipped with a 5972 Mass Selective Detector. Desorption occurred at 200 deg C for 30 seconds followed by a 20 minute chromatographic separation on 30 m x 250 µm HP-1MS column. VOCs were identified using the standard NIST library. Figure 2 shows sample total ion chromatograms (TIC) measured for uninfected piglets (labeled Baseline) and the corresponding TICs observed following infection of the same piglets but prior to the onset of clinical symptoms. The TICs shown in Figure 2 were obtained from either the carboxen/polydimethylsiloxane or the polydimethylsiloxane SPME fibers, both of which gave consistent results.
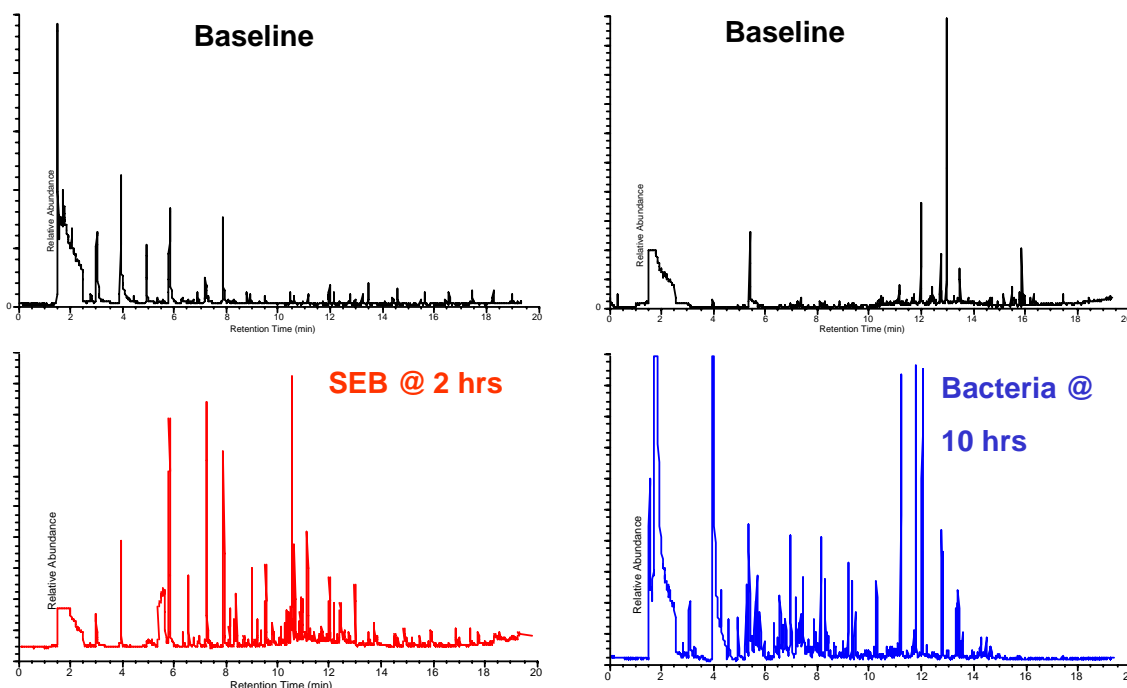

Figure 2. Total ion chromatograms of uninfected piglet breath (Baseline) compared with breath collected after infection with SEB and *A.p.* but prior to the onset of clinical symptoms. Note the air peak below 3 minutes has been truncated for clarity.

Typical VOCs observed with SEB infections included toluene, butyrolactone, methoxy-phenyl-oxime, benzyl alcohol, 3-phenoxy-1-propanal, butylated hydroxy toluene, hexadecane, and 2,6-bis-(1,1 dimethylethyl)-4-(1-oxopropyl) phenol. The variation in the measured amounts of these compounds during the first hour following infection is shown in Figure 3. Volatile markers observed with *A.p.* infections included hexanal, nonanal, benzaldehyde, trimethyl benzene, isopropyl benzene, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, octadecane, docosane, eicosane, tetracosane, dihydrocoumarine, hexadecanoic acid, octadecanoic acid, and dodecylacrylate. It is interesting to note that the VOCs observed for *A.p.* more closely resemble the methylated alkane series found in humans induced by ROS, whereas the VOCs observed for SEB appear to have a different, as yet unknown, etiological origin. Noticeable symptoms did not appear in the SEB-infected animals until 3-4 hours post exposure, and in *A.p.*-infected animals until 13 hours post exposure.

CONCLUSIONS

Based on the GC/MS analysis of the piglet breath samples, we found that: volatile markers appeared several hours before outwardly noticeable physical symptoms of illness; volatile profiles for infected piglets were distinct from the uninfected animals and room background air samples; and the volatile profiles for SEB and *A.p.* were different.

Due to the limited number of piglets examined, the variation in conditions under which samples were collected, and difficulties associated with the use of simple veterinary mask, it was not possible to adequately confirm or quantify a suite of volatile biomarkers that could be monitored for bioagent exposure. In spite of the limitations associated with this preliminary study, the above cited results show great promise for a rapid, non-invasive breath test for BW exposure.
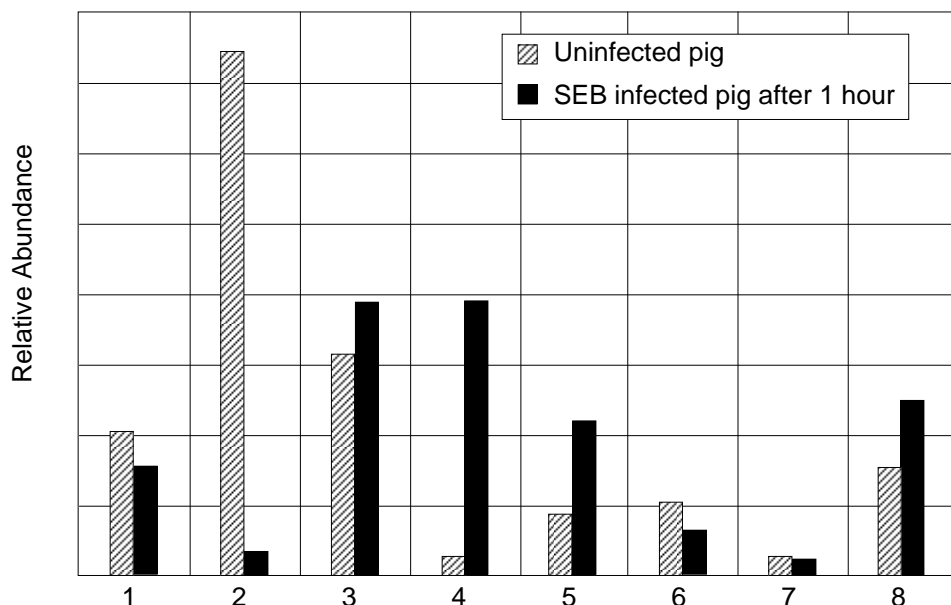

Figure 3: Measured relative amounts of eight potential biomarkers produced in response to SEB infection in piglets. The specific volatile compounds are: (1) toluene, (2) butyrolactone, (3) methoxy-phenyl-oxime, (4) benzyl alcohol, (5) 3-phenoxy-1-propanal, (6) butylated hydroxy toluene, (7) hexadecane, and (8) 2,6-bis-(1,1 dimethylethyl)-4-(1-oxopropyl) phenol.

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